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A Mutation in the Hepatitis E Virus RNA Polymerase Promotes its Replication and Associates with Ribavirin Treatment Failure in Organ Transplant Recipients

Short title: Enhanced HEV fitness in ribavirin failure

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Abbreviations: ALT, alanine aminotransferase; EC_{50}, 50% effective concentration; γGT, γ-glutamyltransferase; HEV, hepatitis E virus; RBV, ribavirin; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; wt, wild-type.

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Abstract

We analyzed blood samples collected from 15 patients with chronic hepatitis E who were recipients of solid-organ transplants. All patients cleared the hepatitis E virus (HEV) except for 2 (nonresponders); 1 patient died. A G1634R mutation in viral polymerase was detected in the HEV RNA of the non-responders; this mutation did not provide the virus with resistance to ribavirin in vitro. However, the mutant form of a subgenomic replicon of genotype 3 HEV replicated more efficiently in vitro than HEV without this mutation, and the same was true for infectious virus, including in competition assays. Similar results were obtained for genotype 1 HEV. The G1634R mutation therefore appears to increase the replicative capacity of HEV in the human liver and hence reduce the efficacy of ribavirin.

Keywords: RNA-dependent RNA polymerase; virulence; drug resistant; mechanism

Hepatitis E virus (HEV) is a feco-orally transmitted RNA virus and a common cause of acute hepatitis worldwide.[1,2] Genotypes 1 and 2 cause water-borne outbreaks in developing countries and exclusively infect humans, whereas genotypes 3 and 4 are zoonotic and consumption of undercooked pig meat is the most documented route of transmission.[1,3] Although HEV infections are often asymptomatic, they can manifest as acute hepatitis, but usually resolve spontaneously. However, genotype 3 infections may evolve to chronicity in immunocompromised patients, e.g. transplant or HIV patients,[4,5] and may lead to cirrhosis, graft loss and death.[6] Ribavirin (RBV) monotherapy is the treatment-of-choice for most patients.[7,8] However, treatment failure has been observed, either as a partial response to ribavirin or viral recurrence after therapy cessation, and is possibly linked to dose reductions because of severe anemia in some cases.[7,8]

We report on 15 solid-organ-transplant patients with chronic hepatitis E (11 with genotype 3c, 3 genotype 3f and 1 genotype 3e). RBV treatment was successful in all but 2 patients (both genotype 3c) who failed to clear the virus (13%, which is comparable to other studies reporting failure rates of 15-18%).[7,8] The first patient was a male heart/kidney-recipient who was treated for 9 months and showed an initial decrease in viral RNA load upon initiation of RBV treatment but never became HEV RNA negative (i.e. below 1000 copies/mL, Figure 1A). Nevertheless, after 4 months of RBV therapy, HEV RNA loads increased again to baseline levels (even before a transient dose reduction because of anemia) and persisted over time. The patient died of hepatic decompensation as described previously.[9] The second patient is a female lung/kidney/bone-marrow-recipient who underwent two consecutive RBV treatments of 4 and 7 months respectively.[10] Although at the end of each treatment period, HEV RNA status was negative, viral RNA was detected again shortly after stopping therapy. ALT levels normalized rapidly, but γGT remained above normal and strongly increased during the second RBV course (Figure 1B). To explore the underlying causes of non-responsiveness to RBV in both patients, complete HEV genome sequences before, during and after treatment were compared. In both patients, a unique G-to-A nucleotide substitution was identified resulting in a G1634R mutation in the C-terminal region of the HEV polymerase. Comparison of HEV sequences in GenBank

revealed that K1634 was the predominant amino acid in genotype 1 and 4, whereas in genotype 3 G1634 was more common (77%) than R1634 (22%, mostly subgenotypes 3e-f) (Figure 1C, Supplementary Table 1).

Since the clinical course and evolution of viral loads was reminiscent of antiviral resistance development, the 1634R mutation was introduced into a genotype 3 replicon and its sensitivity to RBV was compared to that of the wild-type (wt) replicon.[11] No difference in RBV sensitivity was observed, with calculated EC_{50}-values of 5.1±3.7 and 5.1±4.1 µM for G1634 and 1634R respectively (Figure 1D). The 1634R construct however consistently yielded higher luminescence signals than its wt counterpart, suggesting increased viral RNA replication. To further explore this, two hepatoma cell lines were transfected with capped replicon RNA for wt, 1634R and 1634K (predominant in genotype 1). In Huh7 cells, the 1634R construct resulted in a 3.4-fold increase in luminescence signal compared to the G1634 construct (*P*=0.04) and the 1634K construct yielded a 2.7-fold higher signal than wt (*P*=0.07, Figure 1E). In HepG2/C3A cells, both mutants resulted in a significantly increased signal: 1.6-fold for 1634R (*P*=0.02) and 2.1-fold for 1634K (*P*=0.02) (Figure 1E). Similar results were obtained when 10:1, 1:1 or 1:10 mixtures of wt and/or mutant RNA were tested (Supplementary Figure 1).

Next, the impact of the 1634R/K mutations on replication and production of viral progeny of full-length genotype 3 was studied by transfection of Huh7 cells and quantification of released viral RNA. The 1634R and 1634K variants replicated to higher titers than the wt at every time point studied (Figure 2A). When treated with RBV at 10 or 25 µM, replication was partially reduced, but nevertheless the same pattern in relative replication efficiency was observed for the 3 variants. Intracellularly, viral RNA copies per µg RNA were at least 2-fold higher for 1634R/K compared to G1634 (Figure 2B). In untreated HepG2/C3A cells, a comparable pattern was observed, although somewhat less pronounced than in Huh7 cells (Supplementary Figure 2). Treatment of transfected

HepG2/C3A cells with RBV at 10 or 25 µM resulted in a strong inhibition of viral replication so that no differences could be observed.

To compare the fitness of G1634 and 1634R variants, replication was analyzed in direct competition assays. To this end, cells were transfected with mixtures of G1634:1634R full-length RNA in 10:1, 1:1 or 1:10 ratios (90, 50 and 10% G respectively) and were cultured for 20 days. The evolution of the fraction of both variants released into the culture medium was monitored by allele-specific RT-qPCR. The proportion of G1634 was experimentally confirmed to be 12±1, 45±1 and 88±1% in the input RNA and decreased to respectively 2.2±0.4, 17±1 and 61±2% in Huh7 and 2±2, 18±4 and 64±2% in HepG2/C3A cells (Figure 2C). This corresponds to a relative fitness gain for 1634R of 7-9% over 20 days and up to 15% for the first 10 days. A similar pattern was noted in cell lysates (P<0.001, Figure 2D, Supplementary Figure 3). These results provide a strong indication that for genotype 3, 1634R has an increased fitness compared to G1634.

To determine the influence of the 1634 mutations on viral fitness in other genotypes, a genotype 1 replicon in Huh7 cells was employed in which the original K1634 was mutated to 1634R, 1634G, 1634Q or 1634E. As expected, 1634G yielded a decreased signal compared to K1634 (0.7-fold, Figure 2E). Surprisingly, the 1634R mutant also displayed decreased replication (0.8-fold), albeit not statistically significant. A single genotype 1 strain was found with Q1634 (Accession Number JF443723),[12] but in our replicon, 1634Q decreased replication (0.5-fold, P=0.11). Introduction of two other mutations unique to this strain yielded replication-impaired or lethal phenotypes (Supplementary Figure 4). Finally, the 1634E mutant displayed a significantly reduced replication (0.4-fold, P=0.049) as expected, given that this is a negatively-charged amino acid, contrary to the positively-charged R and K.

In conclusion, this is the first reported virulence mutation in the HEV genome that was confirmed *in vitro*. The increased replication capacity of the mutant may have contributed to the persistent disease courses despite RBV treatment, although other patient- and virus-related factors could have

contributed as well.[6,13] It may be interesting to assess the possible use of position 1634 as a prognostic marker and accordingly to adjust dose and duration of ribavirin therapy based on the presence of the G1634R variant.

Figure legends

Figure 1. RBV treatment failure is associated with a G1634R mutation. (*A*-*B*) Clinical course and sequencing results for patients experiencing RBV failure; arrow indicates RBV dose reduction. (*C*) Prevalence of G, R and K at position 1634 per genotype. (*D*) G1634R does not alter RBV sensitivity. (*E*) Increased luminescence read-out in 1634R/K compared to G1634 in Huh7 and HepG2/C3A cells. **P*<0.05, ***P*<0.01.

Figure 2. 1634R/K has an increased fitness compared to G1634. (*A*) Kinetics of released genotype 3 RNA indicate increased replication of 1634R/K strains compared to G1634, both in untreated or RBV-treated cells. (*B*) Intracellular viral RNA reveals a similar pattern. (*C*) Mixtures of G1634 and 1634R full-length RNA were transfected into Huh7 or HepG2/C3A cells and the fraction of wt G1634 in released viral RNA was monitored, indicating outcompeting of G1634 by 1634R. (*D*) Comparing input RNA with intracellular viral RNA 20 days post transfection shows a similar decrease in the G1634 fraction. (*E*) In a genotype 1 replicon, replication is decreased for 1634G/R/Q/E compared to the wt K1634 in Huh7 cells. **P*<0.05, ****P*<0.001.

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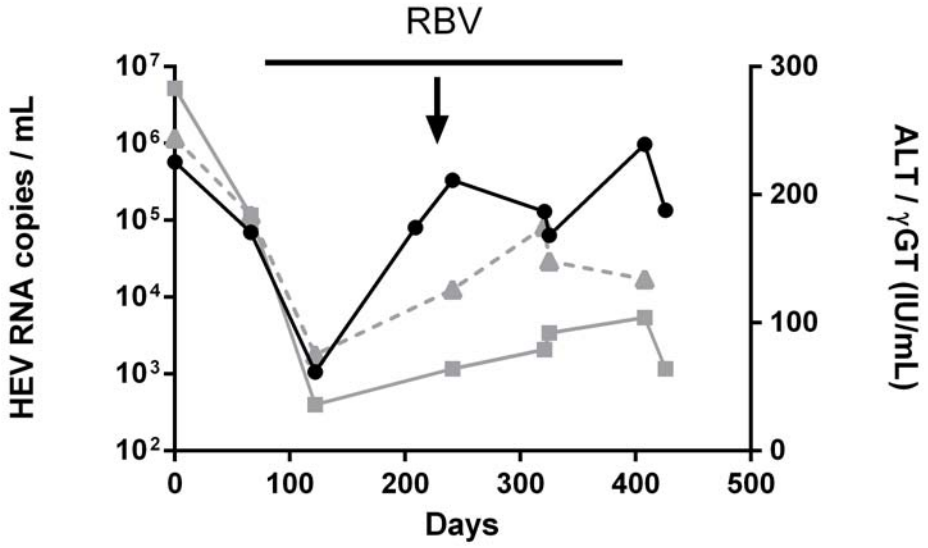

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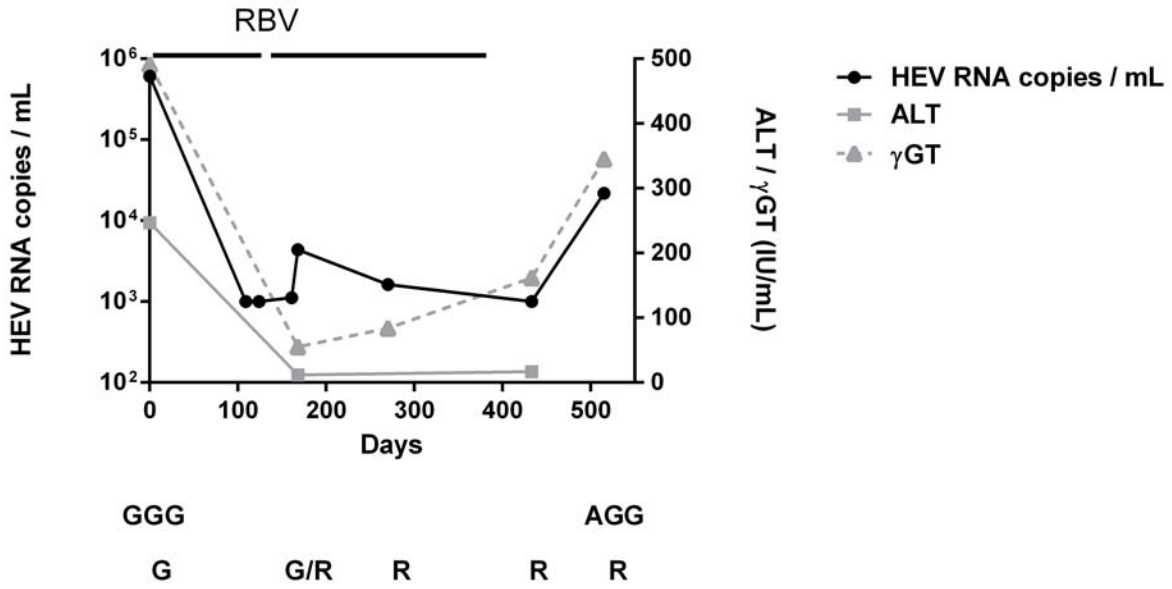
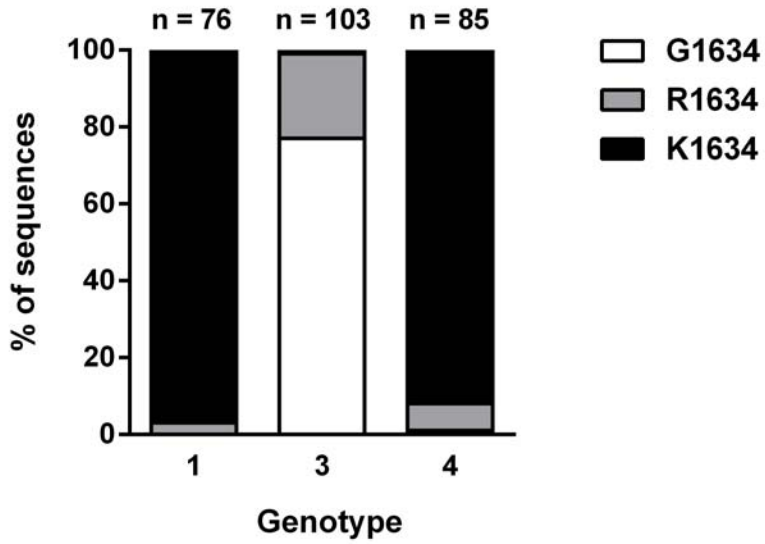
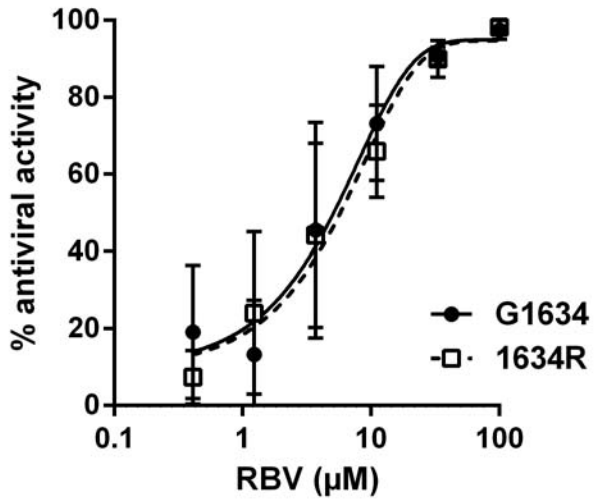
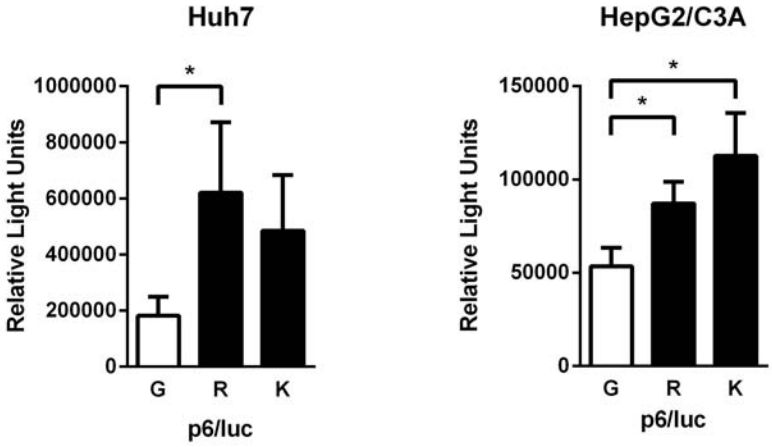
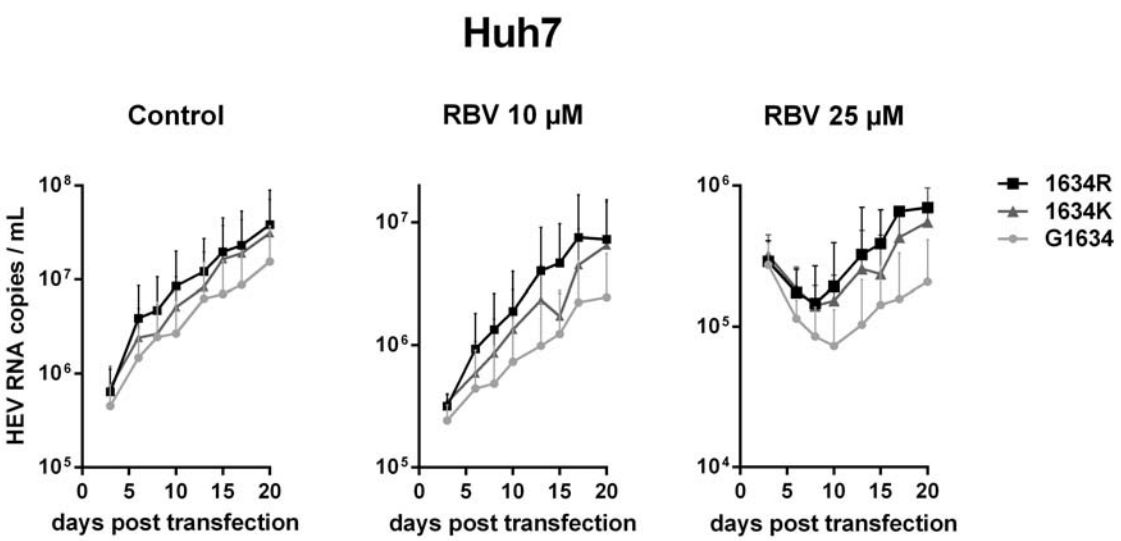
RdRp 1634	G	G	G	R	R	R	R	R

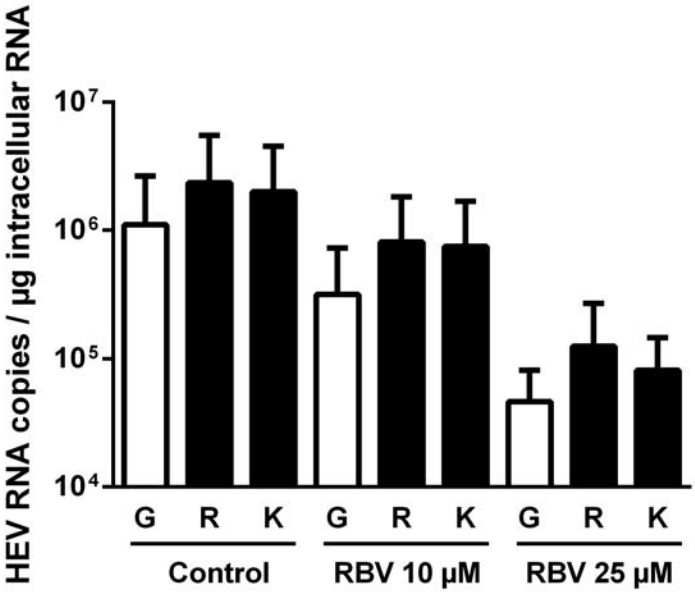


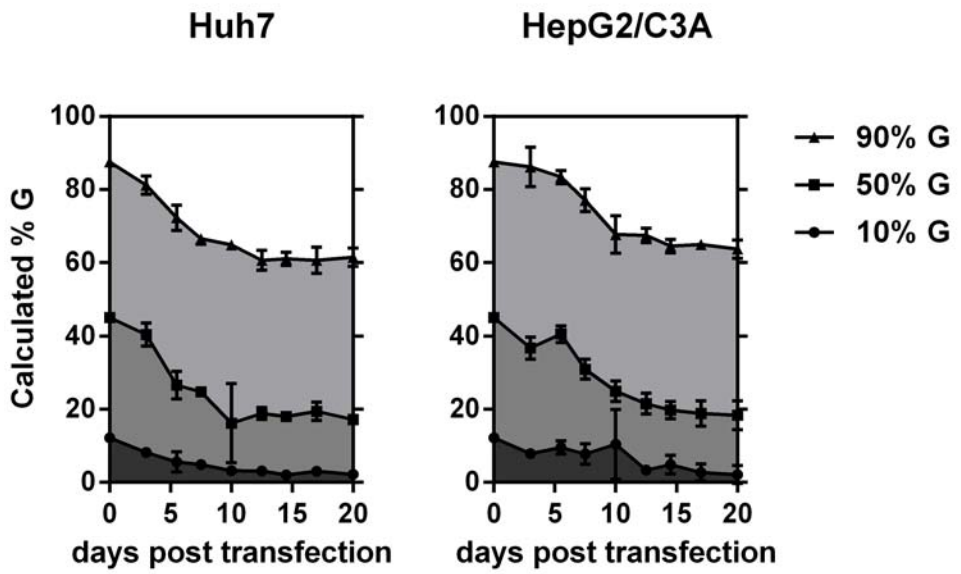


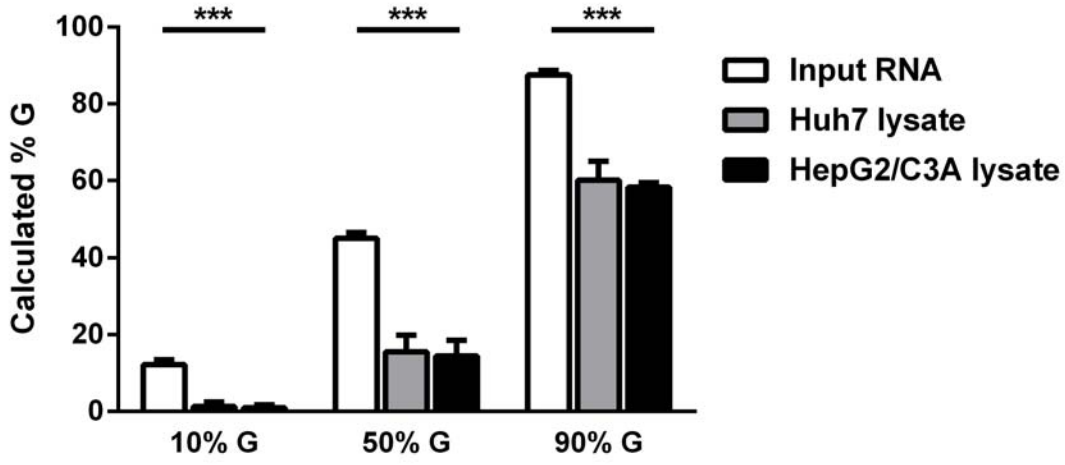


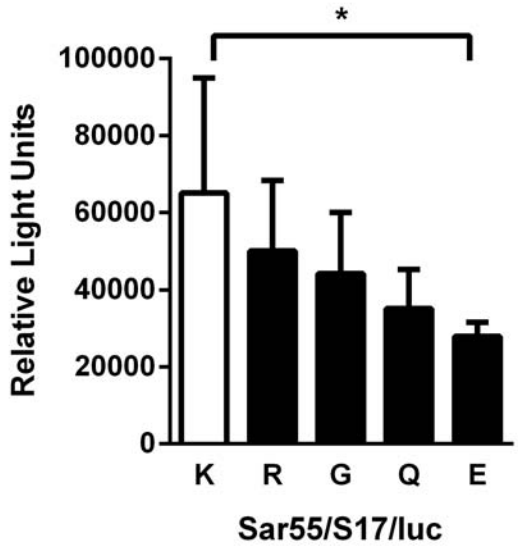












Supplementary materials and methods

Study subjects

All patients were recruited at Hannover Medical School between 2008 and 2014. Serum samples were collected from 15 solid organ-transplanted (SOT) patients who developed chronic hepatitis E and received ribavirin (RBV) treatment. All patients were infected with genotype 3 HEV. Serum samples were collected before (I), during (II) and after RBV treatment (III). At least one serum sample from each time point (I-III) was used for further studies. Written informed consent was obtained from each patient included in this study. The study protocol conformed to the ethical guidelines of the Institutional Review Committee.

RBV (Rebetol® or Copegus®) was administered orally twice daily with an initial daily dose of 600-1000 mg, depending on the patients' hemoglobin level and comorbidities.[1] Dose reductions were performed if hemoglobin levels declined and/or patients developed symptoms associated with anemia. At each visit, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltranspeptidase (γGT) as well as HEV serology and HEV RNA viremia were determined. Anti-HEV status was determined using Wantai HEV IgG (Beijing, China). HEV RNA from serum was quantified by one-step RT-qPCR as described.[2] All patients cleared HEV RNA by the end of treatment, except for 2 chronically infected transplant recipients (both infected with genotype 3c).

Extraction and sequencing of HEV RNA from serum samples

Total RNA was extracted from 200 µl of serum or EDTA-plasma using Cobas AmpliPrep total nucleic acid isolation kit (Roche, Basel, Switzerland). Total RNA concentration was measured using the NanoVue Plus spectrophotometer (GE Healthcare Life Sciences, Little Chalfont, UK). cDNA was synthesized from 4 to 8 µL of purified total RNA using the SuperScript III first-strand synthesis system (Life Technologies, Carlsbad, CA) with the external reverse primer from each set (set1-4 A2 (ex)) at a final concentration of 2 µM (see section Primers and probes). A touchdown nested PCR (n-PCR) with 4 sets of specific external and internal primer pairs listed in the section Primers and probes was used

to amplify the coding regions of the HEV genome. The first PCR round was carried out with TaKaRa Ex Taq Hot Start Version (Dalian, China) using 8 µL of synthesized cDNA and an external primer pair at a final concentration of 1 µM each in a 50 µL reaction with 18 cycles of 30 s at 94°C, 45 s at 62°C with a reduction of 0.5°C/cycle and 1 min/kb at 72°C, followed by 14 cycles of 30 s at 94°C, 45 s at 53°C and 1 min/kb at 72°C. A final extension of 10 min at 72°C followed the final cycle. The second PCR round was carried out using internal primer pairs and 5 µL of the first-round PCR product with identical amplification parameters to the first round. The resulting amplicons were separated by agarose gel electrophoresis and purified using Qiaquick gel extraction kit (Qiagen, Hilden, Germany) and concentrations were measured using NanoVue Plus spectrophotometer (for Sanger sequencing).

Sanger sequencing and analysis of HEV coding regions

The purified PCR products were sequenced commercially (GATC Biotech, Konstanz, Germany) in both forward and reverse directions using the internal primer pairs from set 1-4 (see section Primers and probes) on an automatic DNA sequencer (Sanger ABI 3730xl). To identify nucleotide and amino acid variations between different time points (before, during and after treatment) the nucleotide sequences from all sets were assembled using Sequencher 4.9 software by Gene Codes (Ann Arbor, MI).

Cells, viruses and replicons

Huh7 cells (a kind gift from Ralf Bartenschlager, University of Heidelberg, Germany) and HepG2/C3A cells (a kind gift from Luc Verschaeve, Scientific Institute of Public Health, Brussels, Belgium) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco) in a humidified 5% CO2 incubator at 37°C.

Genotype 3 full-length and reporter replicon viral RNA was derived from plasmids encoding Kernow-C1 p6 (GenBank accession number JQ679013) and Kernow-C1 p6/luc HEV strains respectively.[3] Genotype 1 replicon RNA was derived from Sar55/S17/luc-encoding plasmid.[4] Both were kind gifts

from Suzanne U. Emerson (NIAID, NIH, Bethesda, MD). Amino acid numbering is according to NCBI reference sequence NP_056779.

Site-directed mutagenesis and plasmid preparation

Mutations were introduced by PCR amplification of overlapping fragments with specifically mutated primers. To introduce the 1634R mutation into the Kernow-C1 p6/luc replicon, PCR amplifications were performed with primer pairs KC1p6-*Afl*II-5'f + KC1p6-1634Rr and KC1p6-1634Rf + KC1p6/luc-*Nru*I-3'r (see section Primers and probes for primer sequences). Consequently both fragments were combined in a fusion PCR with primers KC1p6-*Afl*II-5'f and KC1p6/luc-*Nru*I-3'r. PCRs were performed with KAPA HiFi HotStart ReadyMix PCR kit (Kapa biosystems, Wilmington, MA). The resulting fragment was digested with *Afl*II, phosphorylated and ligated into *Afl*II- and *Nru*I-digested Kernow-C1 p6/luc plasmid. Similarly, primer pairs KC1p6-*Afl*II-5'f + KC1p6-1634Kr and KC1p6-1634Kf + KC1p6/luc-*Nru*I-3'r were used to introduce the 1634K mutation. To mutate the full-length Kernow-C1 p6 plasmid, PCRs were performed with the same primer sets except KC1p6-*Pml*I-3'r was used as the ultimate 3'-end primer. The resulting fusion fragment was digested with *Afl*II, phosphorylated and ligated into *Afl*II- and *Pml*I-digested Kernow-C1 p6 vector.

To mutate the genotype 1 Sar55/S17/luc construct (wild-type (wt): K1634), PCRs were performed with primer pairs SarLuc-*Sfi*I-5'f + SarLuc-1634Gr and SarLuc-1634Gf + SarLuc-*Nhe*I-3'r (1634G), SarLuc-*Sfi*I-5'f + SarLuc-1634Rr and SarLuc-1634Rf + SarLuc-*Nhe*I-3'r (1634R), SarLuc-*Sfi*I-5'f + SarLuc-1634Er and SarLuc-1634Ef + SarLuc-*Nhe*I-3'r (1634E), SarLuc-*Sfi*I-5'f + SarLuc-1634Qr and SarLuc-1634Qf + SarLuc-*Nhe*I-3'r (1634Q), SarLuc-*Sfi*I-5'f + SarLuc-1346Gr and SarLuc-1346Gf + SarLuc-*Nhe*I-3'r (1346G), SarLuc-*Sfi*I-5'f + SarLuc-1498Pr and SarLuc-1498Pf + SarLuc-*Nhe*I-3'r (1498P), SarLuc-*Sfi*I-5'f + SarLuc-1346Gr and SarLuc-1346Gf + SarLuc-1498Pr and SarLuc-1498Pf + SarLuc-*Nhe*I-3'r (1346G + 1498P). Fragments were combined in fusion PCRs with primers SarLuc-*Sfi*I-5'f and SarLuc-*Nhe*I-3'r and digested with *Nhe*I and *Sfi*I. The resulting fragments were ligated into *Nhe*I- and *Sfi*I-digested Sar55/S17/luc vector.

E. coli Top10 cells (Life Technologies) were transformed with ligated plasmids. Kernow-C1-related constructs were cultured in 500 mL of Super Broth with ampicillin and maxiprepped (Plasmid maxi kit, Qiagen). Sar55-related plasmids were cultured in 100 mL of LB medium with ampicillin and midiprepped (NucleoBond Xtra midi kit, Macherey-Nagel, Düren, Germany). The cloned regions in each of the constructs were sequenced to ensure that no additional mutations had been introduced.

In vitro transcription and capping

Viral RNA was *in vitro* transcribed from *Mlu*I- (Kernow-related) or *Bgl*II- (Sar55-related) linearized plasmid DNA with the RiboMAX Large Scale RNA Production System-T7 (Promega, Madison, WI) and capped with the ScriptCap m7G capping system (Cellscript, Madison, WI). A firefly luciferase-based transfection control was generated as described.[5] Nucleic acid concentrations were determined by spectroscopy (Nanodrop ND-1000, Thermo Fischer Scientific, Waltham, MA).

Antiviral assay

Luminescence-based antiviral assays were performed essentially as described.[5] RBV [1-(β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide (Virazole®)] was purchased from ICN Pharmaceuticals (Costa Mesa, CA). A stock solution was prepared in DMSO and stored at 4°C.

Replicon assays

Huh7 and HepG2/C3A cells were seeded into 6-well plates at $2x10^5$ cells per well and transfected with capped RNA transcripts (1 µg per well) 24h later using Lipofectin (Life Technologies) according to the manufacturer's instructions. After 72h of incubation at 35°C, *Gaussia* luciferase activity was measured in 20 µL of culture medium with the *Renilla* luciferase assay system (Promega).

For replicon competition assays, Huh7 and HepG2/C3A cells were seeded into 12-well plates at $8x10^4$ cells per well. RNA was produced for each wt and mutant Kernow-C1 p6/luc replicon and 2 types of uncapped RNA were mixed in 10:1, 1:1 and 1:10 ratios. Mixtures were capped and 0.4 µg per well was transfected into cells 24h after seeding with Lipofectin. After 72h of incubation at 35°C, *Gaussia* luciferase activity was determined.

Full-length HEV replication kinetics and competition assays

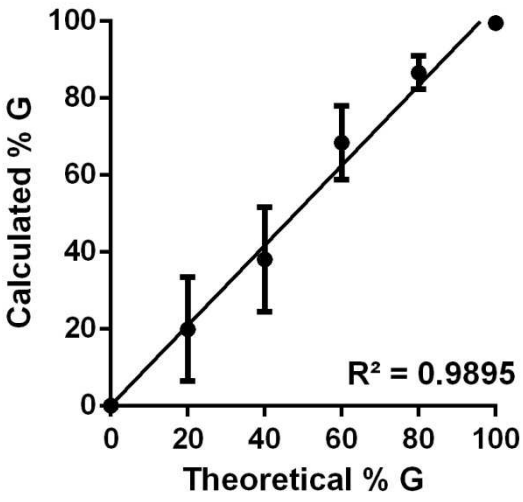
To determine the growth kinetics of wt and mutant full-length HEV (G1634, 1634R and 1634K), Huh7 and HepG2/C3A cells were seeded into 6-well plates at $2x10^5$ cells per well and transfected with capped RNA transcripts for Kernow-C1 p6 wt or mutants (1 µg per well). DMSO (0.125%) or RBV at 10 or 25 µM were included in the culture medium. One mL of the medium was removed every 2-3 days, stored at -80°C until RNA extraction and 1mL of fresh medium (with DMSO or RBV) was added to each well. After 20 days, cell layers were lysed and intracellular RNA was extracted with the Qiagen RNeasy kit. To extract viral RNA from culture medium, RNase A (Promega) was added to 100 µL of thawed medium to a final concentration of 200 ng/mL and incubated at room temperature for 5' to reduce the amount of residual in vitro transcripts from RNA transfection. Viral RNA was extracted with the NucleoSpin RNA virus kit (Macherey-Nagel) and quantified by reverse transcription quantitative PCR (RT-qPCR).

For infectious virus competition assays, Huh7 and HepG2/C3A cells were seeded into 6-well plates at $2x10^5$ cells per well. RNA was produced for Kernow-C1 p6 wt and 1634R mutant and was mixed in 10:1, 1:1 and 1:10 ratios. Mixtures were capped and transfected with Lipofectin into cells 24h after seeding. One mL of the medium was removed every 2-3 days, stored at -80°C until RNA extraction and 1mL of fresh medium was added to each well. RNase treatment and RNA extractions were performed as described above. RNA extracts were analyzed by allele-specific multiplex RT-qPCR. Input RNA mixtures and RNA extracts from cell lysates were subjected to RT-PCR with primers KC1p6-*Afl*II-5'f and KC1p6-*Pml*I-3'r (Onestep RT-PCR kit, Qiagen) and subsequent Sanger sequencing with primer KC1p6-*Afl*II-5'f (BigDye Terminator v3.1 cycle sequencing kit, Life Technologies) for semiquantitative assessment of the G1634 : 1634R ratio. For calculation of relative fitness gains, the fitness of G1634 and 1634R strains is considered 1 and 1+*s* respectively with *s* being the relative fitness gain. *s* is calculated as described.[6]

RT-qPCR

Quantification of total HEV RNA was performed essentially as described with primers HEVqf and HEVqr and probe HEVqp.[5] For absolute quantification of intracellular viral RNA, copy numbers were normalized to total RNA per sample as determined by spectroscopy.

Allele-specific multiplex RT-qPCR was performed with primers KC1p6-asqf and KC1p6-asqr. As probes, KC1p6-asqpG and KC1p6-asqpR were used (see section Primers and probes). Reactions were performed with One-Step qRT-PCR mix (Eurogentec, Seraing, Belgium) in a final volume of 25 µL containing 375 nM of each primer, 125 nM of each probe and 5 µL of RNA sample using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) under following conditions: 30 min at 48°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Data were analyzed with ABI PRISM 7500 SDS software (version 1.3.1, Applied Biosystems). For absolute quantification, standard curves were generated using 10-fold dilutions of the cloned target cDNA. Concentrations of DNA standards were calculated by spectroscopy (for regular RT-qPCR) or RT-qPCR (for allele-specific multiplex RT-qPCR). In order to validate the allele-specific multiplex RT-qPCR, mixtures of G1634 and 1634R RNA were made in known ratios (0, 20, 40, 60, 80 and 100% wt) and analyzed. Results show a strong correlation between expected and experimentally obtained fractions of wt ($R^2 = 0.9895$, n = 3).



Primers and probes

Primers for analysis of clinical samples		
Set	**Primer**	**Sequence**
1	Set1-S1 (ex)	5’-AGGCTCCTGGCATTACTACTG-3’
	Set1-S2 (in)	5’-GCCTTGGCGAATGCTGTG-3’
	Set1-A1 (in)	5’-GGCCGGGGATGTARTCACG-3’
	Set1-A2 (ex)	5’-CGGCACTGRGCATARAACTG-3’
2	Set2-S1 (ex)	5’-ATGACATACCTYCGTGGYATTAG-3’
	Set2-S2 (in)	5’-GTYGCYAATGAGGGKTGGAA-3’
	Set2-A1 (in)	5’-TTGTGGTCTCTGTRAARGTRGCRCCCT-3’
	Set2-A2 (ex)	5’-CRGCCGTRGCTATAATTGTRGTCT-3’
3	Set3-S1 (ex)	5’-GYTTTGCGGCCTTYACACCYCAYAC-3’
	Set3-S2 (in)	5’-CGYCGTGTTGTKATTGAYGAGGC-3’
	Set3-A1 (in)	5’-GRCCRGCAAAHCGCACYACAT-3’
	Set3-A2 (ex)	5’-AACACARACCTGCGCRACATTCGT-3’
4	Set4-S1 (ex)	5’-CCTGGYACCCTYCTYTGGAAYAC-3’
	Set4-S2 (in)	5’-TGGGYTGTATGCYGGTGTGGTRGT-3’
	Set4-A1 (in)	5’-CAGCCGACGAAATCAATTCTG-3’
	Set4-A2 (ex)	5’-CCCTTATCCTGCTGYGCATT-3’

Primers for mutagenesis and *in vitro* studies		
Primer name	**Sequence**	
KC1p6-*Afl*II-5’f	5’-TGATCACTTAAGGGTTTCTGGAAGAAGC-3’	
KC1p6-1634Rr	5’-TCAA**CCT**TCGAAGGAAATCACAAACAG-3’	
KC1p6-1634Kr	5’-TCAA**CTT**TCGAAGGAAATCACAAACAG-3’	
KC1p6-1634Rf	5’-CTGTTTGTGATTTCCTTCGA**AGG**TTGACGAACG-3’	
KC1p6-1634Kf	5’-CTGTTTGTGATTTCCTTCGA**AAG**TTGACGAACG-3’	
KC1p6/luc-*Nru*I-3’r	5’-CGAAGTTGCTGGCCACGGCCAC-3’	
KC1p6-*Pml*I-3’r	5’-GTGAATCAACATCAGGTACAGGGGCTG-3’	
SarLuc-*Sfi*I-5’f	5’-AGATCTGGCCGTTATGGCCGCCGCACAAAG-3’	
SarLuc-1634Gr	5’-TGAG**CCC**GCGGAGAAAATCACTCACAG-3’	
SarLuc-1634Rr	5’-TGAG**CCT**GCGGAGAAAATCACTCACAG-3’	
SarLuc-1634Er	5’-TGAG**CTC**GCGGAGAAAATCACTCACAG-3’	
SarLuc-1634Qr	5’-TGAG**CTG**GCGGAGAAAATCACTCACAG-3’	
SarLuc-1634Gf	5’-CTGTGAGTGATTTTCTCCGC**GGG**CTCACGAATG-3’	
SarLuc-1634Rf	5’-CTGTGAGTGATTTTCTCCGC**AGG**CTCACGAATG-3’	
SarLuc-1634Ef	5’-CTGTGAGTGATTTTCTCCGC**GAG**CTCACGAATG-3’	
SarLuc-1634Qf	5’-CTGTGAGTGATTTTCTCCGC**CAG**CTCACGAATG-3’	
SarLuc-1346Gr	5’-GCTCGTACAATTC**CCC**GGTTGTAACC-3’	
SarLuc-1346Gf	5’-GGTTACAACC**GGG**GAATTGTACGAGC-3’	
SarLuc-1498Pr	5’-GCAGAATCCACGC**AGG**CCTTATAAGGTGG-3’	
SarLuc-1498Pf	5’-CCACCTTATAAGG**CCT**GCGTGGATTCTGC-3’	
SarLuc-*Nhe*I-3’r	5’-CAAGCAATGCTAGCACAGAGTGG-3’	
HEVqf	5’-GGTGGTTTCTGGGGTGAC-3’	
HEVqr	5’-AGGGGTTGGTTGGATGAA-3’	
HEVqp	5’-6FAM-TGATTCTCAGCCCTTCGC-MGBNFQ-3’	(from Life Technologies)
KC1p6-asqf	5’-TGCTGAGCAGCTACGTC-3’	
KC1p6-asqr	5’-GGGCTAACTCCATAGACACG-3’	

KC1p6-asqpG	5'-6FAM-TCCTTCGAG-ZEN-GGTTGACGA-IBFQ-3'
KC1p6-asqpR	5'-HEX-TCCTTCGAA-ZEN-GGTTGACGAA-IBFQ-3'

Restriction sites (or partial restriction sites) are underlined and mutated codons are in bold-type. All primers for quantification and analysis of clinical samples were purchased at Eurofins MWG Operon (Germany), while primers and probes for *in vitro* studies were purchased at Integrated DNA Technologies (Coralville, IA), unless otherwise indicated. K=G/T/U, M=A/C, R=A/G, S=C/G, W=A/T, Y=C/T/U, N=A/T/G/C, H=A/T/C, V=A/G/C, D=A/T/G. 6FAM, 6- fluorescein amidite; HEX, hexachlorofluorescein; IBFQ, Iowa black fluorescent quencher; MGBNFQ, minor groove-binding non-fluorescent quencher; ZEN, internal quencher.

Statistical analysis

Results for all experiments are derived from at least 3 independent experiments and were analyzed by two-tailed Student *t* test.

Supplementary references

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2. Jothikumar N, et al J Virol Methods 2006;131:65-71.
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4. Nguyen HT, et al. J Virol 2014;88:868-877.
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Author names in bold designate shared co-first authorship.

Genotype	Number of sequences	Sequences with G1634 (%)	Sequences with R1634 (%)	Sequences with K1634 (%)
1	76	0 (0)	2 (3)	73 (96)
2	1	0 (0)	1 (100)	0 (0)
3	103	79 (77)	23 (22)	1 (1)
4	85	1 (1)	6 (7)	78 (92)
Total	265	80 (30)	32 (12)	152 (57)

Supplementary Table 1 – Prevalence of G, R and K at position 1634 per genotype: HEV sequences from genotypes 1 to 4 for which a sequence of the C-terminal RdRp was available were used to calculate the prevalence of each amino acid at position 1634. Sequences obtained through BLASTP alignment, genotyping based on phylogenetic grouping with known genotype sequences (ClustalW2).

Supplementary Figure legends

Supplementary Figure 1. 1634R/K display increased replication compared to G1634 in a genotype 3 replicon assay when mixtures of G1634 : 1634R (G:R), G1634 : 1634K (G:K) and 1634R : 1634K (R:K) in 10:1, 1:1 or 1:10 ratios are transfected into Huh7 (*A*) or HepG2/C3A (*B*) cells. For Huh7, significant differences were found between 10:1 and 1:10 mixtures of G:R (p=0.006) and G:K (p=0.04). Results in HepG2/C3A cells were fully in line with this. **P*<0.05, ***P*<0.01.

Supplementary Figure 2. Effect of G1634R/K on full-length genotype 3 HEV replication in HepG2/C3A cells. (*A*) Kinetics of released viral RNA after transfection in untreated HepG2/C3A cells indicate an increased replication for 1634R/K strains compared to wt G1634. Treatment with RBV at 10 or 25 µM results in a strong inhibition of viral replication. As a result, no differences in replication can be observed. (*B*) Intracellular viral RNA from transfected HepG2/C3A cells reveals a similar pattern as in released RNA. The higher RNA levels observed in cells treated with 25 µM of RBV compared to 10 µM are due to cytostatic effects on HepG2/C3A cells at this concentration and thus a strong decrease in total cellular RNA.

Supplementary Figure 3. Sequencing of input and intracellular viral RNA from competition assay. Mixtures of full-length G1634 and 1634R HEV RNA were transfected into Huh7 and HepG2/C3A cells. RT-PCR and subsequent Sanger sequencing were performed on input and intracellular viral RNA 20 days post transfection to compare relative amounts of G and R at position 1634 (marked with arrow). A gradual increase in the fraction 1634R can be observed in all mixtures in both cell lines.

Supplementary Figure 4. 1634Q and other unique mutations in a genotype 1 HEV replicon. Viral replication is impaired for 1634Q compared to wt K1634 and even further for a combination of 1346G+1634Q (GQ). The combinations 1498P+1634Q (PQ) and 1346G+1498P+1634Q (GPQ) result in a lethal phenotype. **P*<0.05.